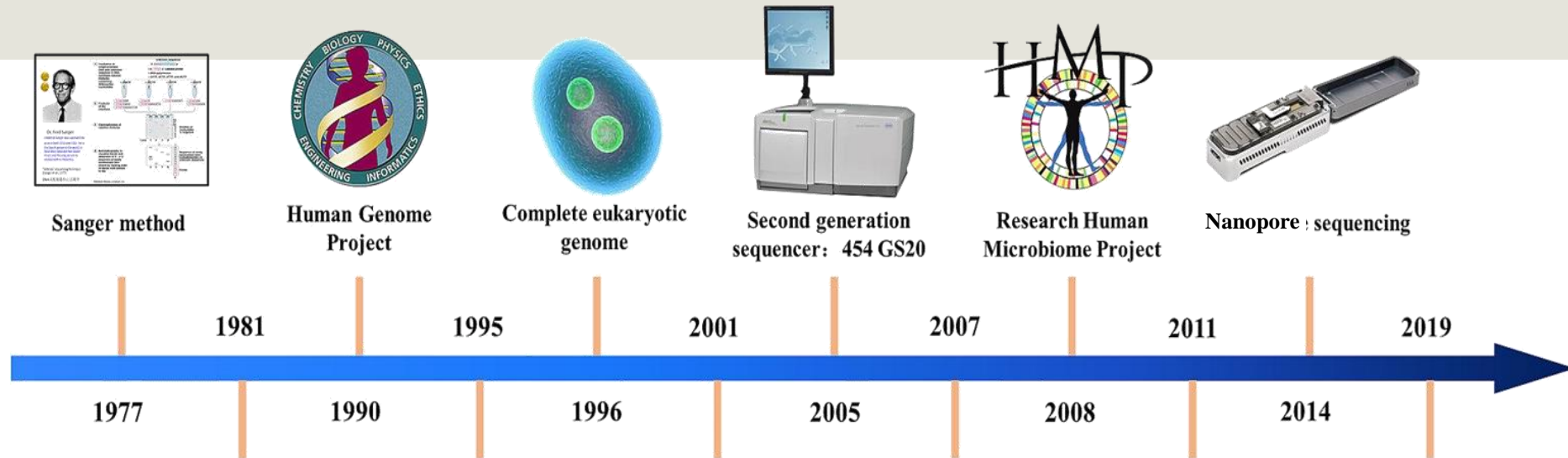


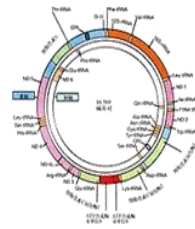
Rio Grande RS 2023

[illegible]

Sequencing through time...



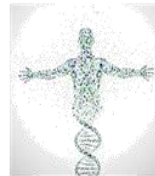
Human mitochondrial genome sequence



Complete cell genome



Complete the Human Genome Project



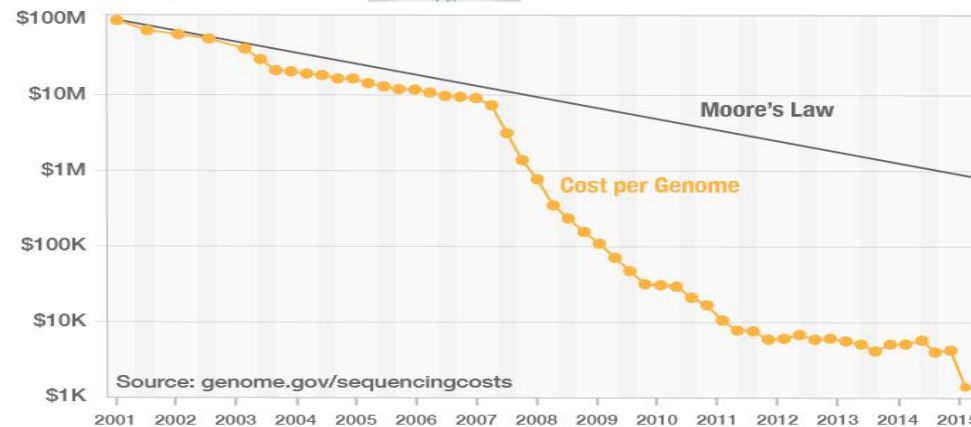
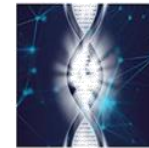
Second generation sequencer: Genetic Analyzer 2



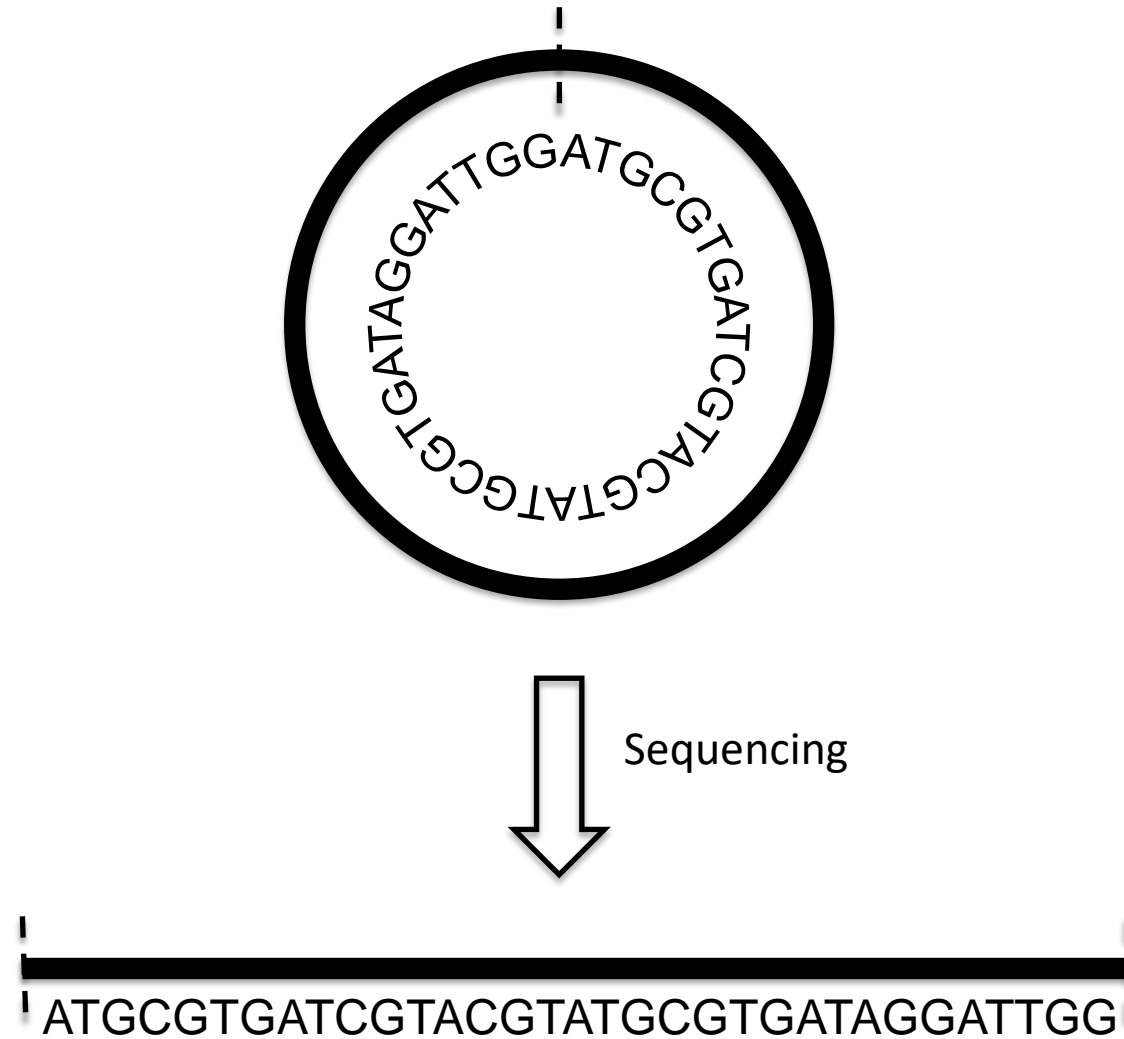
Third generation sequencer: PacBio RS



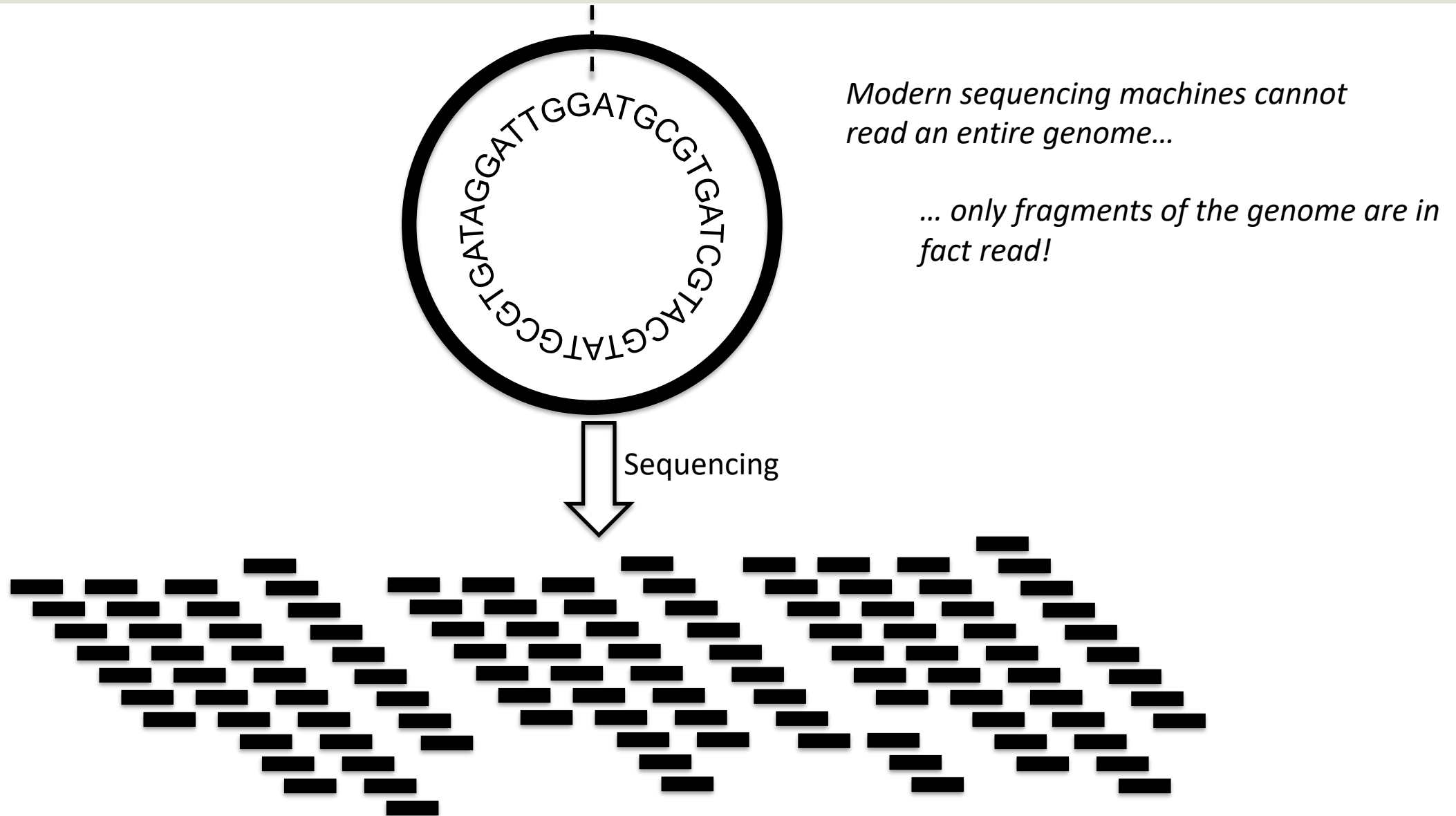
The third stage Human microbiome project







Genome Sequencing: Ideal situation...



Genome Sequencing: the hard reality...



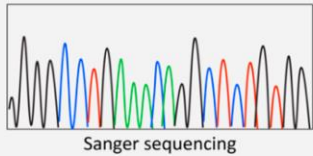
NGS Platforms: An overview ...

				
Read Length (bp)	50-300	200-400	10000-40000	1Mbp
Output (Gb)	6000	0,05-1	0,5-1	5-40
Cost / Million bp (USD)	0,05-0,15	1	0,13-0,60	variable
Accuracy	99.9%	99.6%	87%	92-97%
Time per run	1-11d	2h	30min-20h	1min-48h

Sanger Cost per MB: 2400USD

Next Generation Sequencing

A First generation sequencing



Targeted sequencing

Advantages

- + Accuracy
- + Costs (< 20 amplicons)
- + Turnaround time

Disadvantages

- Capacity
- Costs (>20 amplicons)
- Throughput

B Next generation sequencing



Illumina whole
exome sequencing



Illumina whole
genome sequencing

Short read sequencing

Advantages

- + Applicability
- + Costs
- + Throughput

Disadvantages

- Coverage and mapping
- Data interpretation
- Structural variant detection

C Third generation sequencing



SMRT sequencing



Nanopore sequencing

Long read sequencing

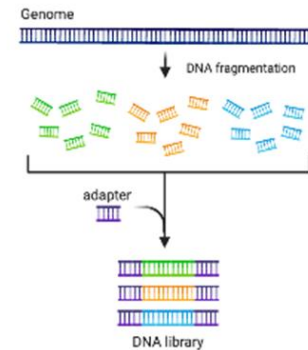
Advantages

- + Coverage and mapping
- + De novo assembly
- + Structural variant detection

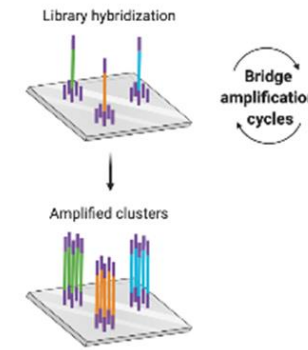
Disadvantages

- Accuracy
- Costs
- Library preparation

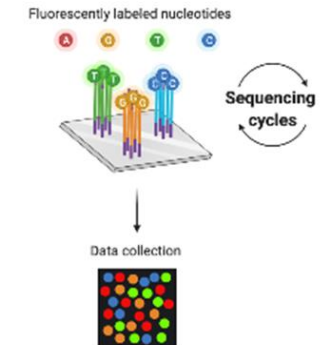
1 Library preparation



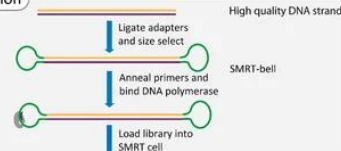
2 DNA library bridge amplification



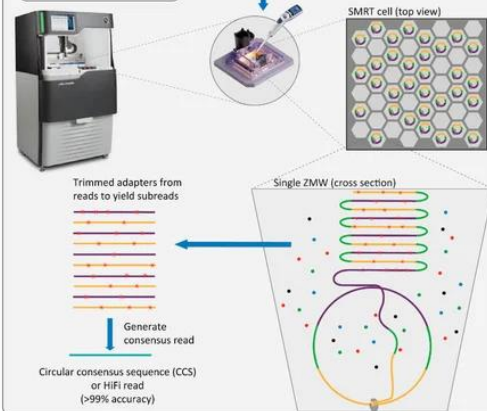
3 DNA library sequencing



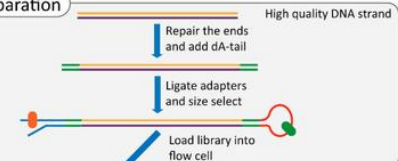
A Library preparation



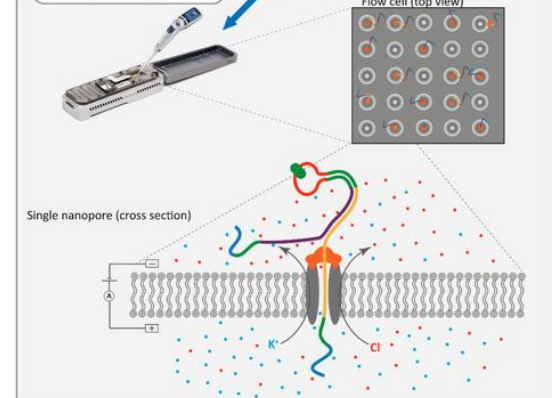
B Sequencing process



A Library preparation



B Sequencing process



Illumina: Sequencing-by-synthesis

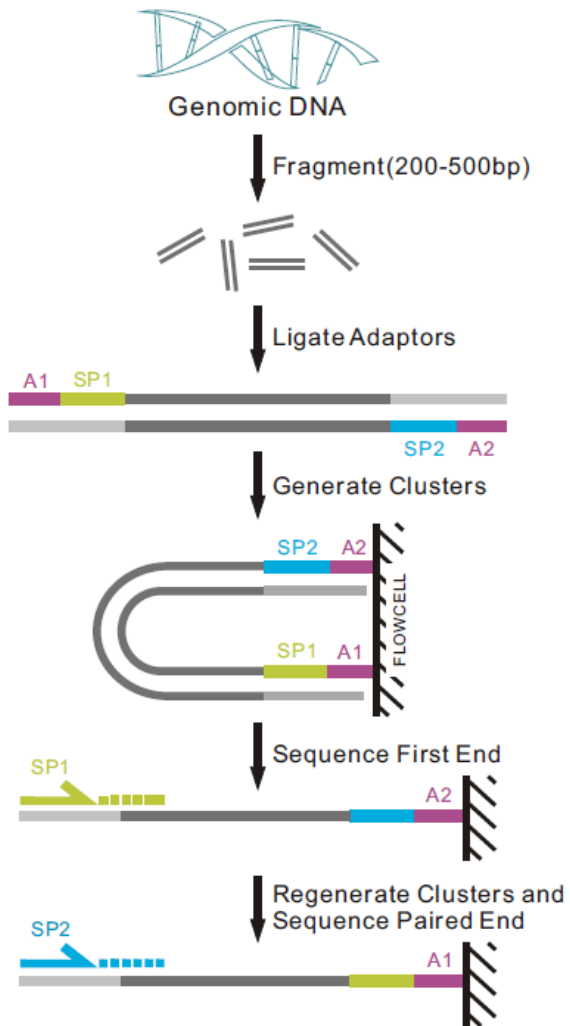
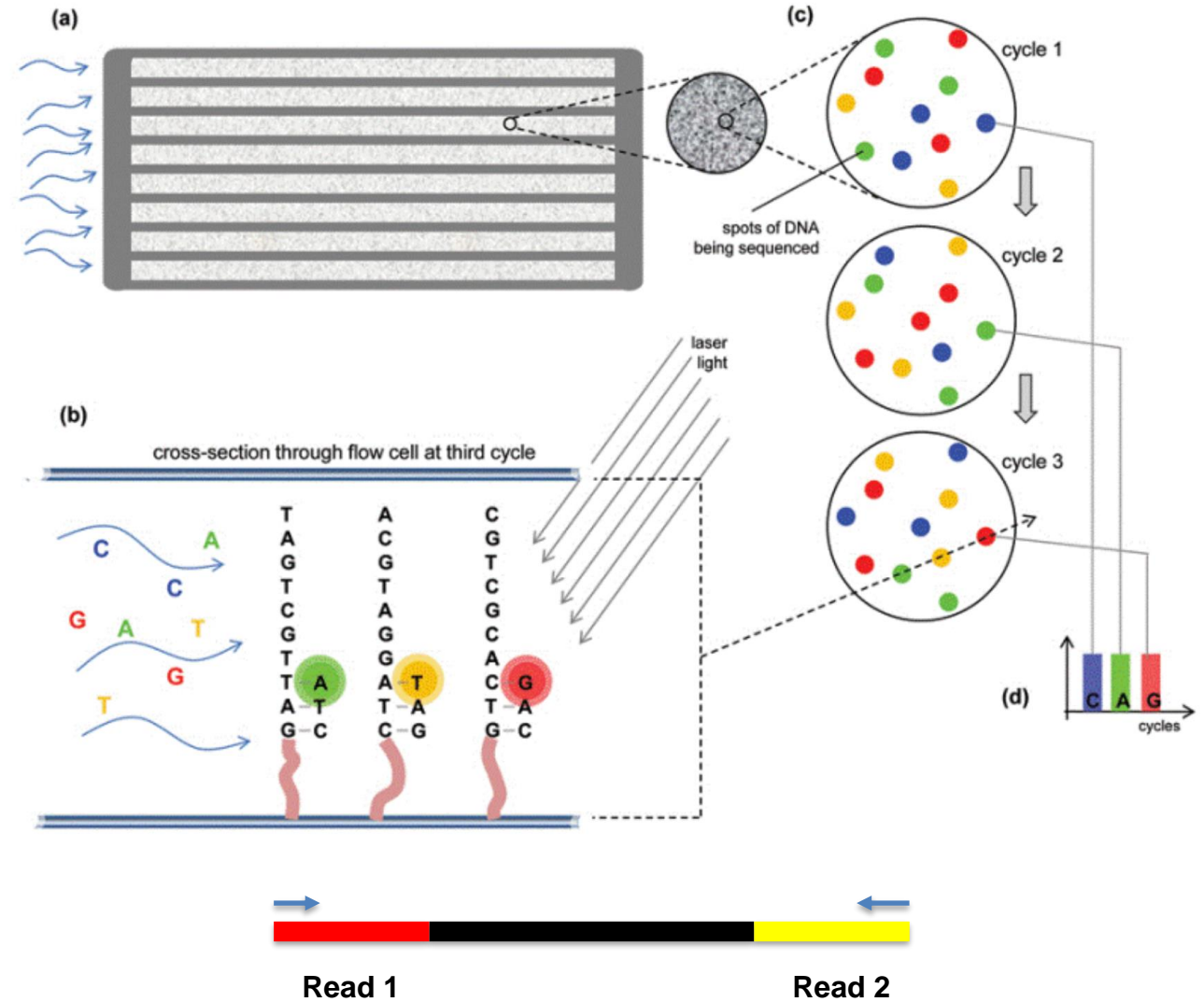
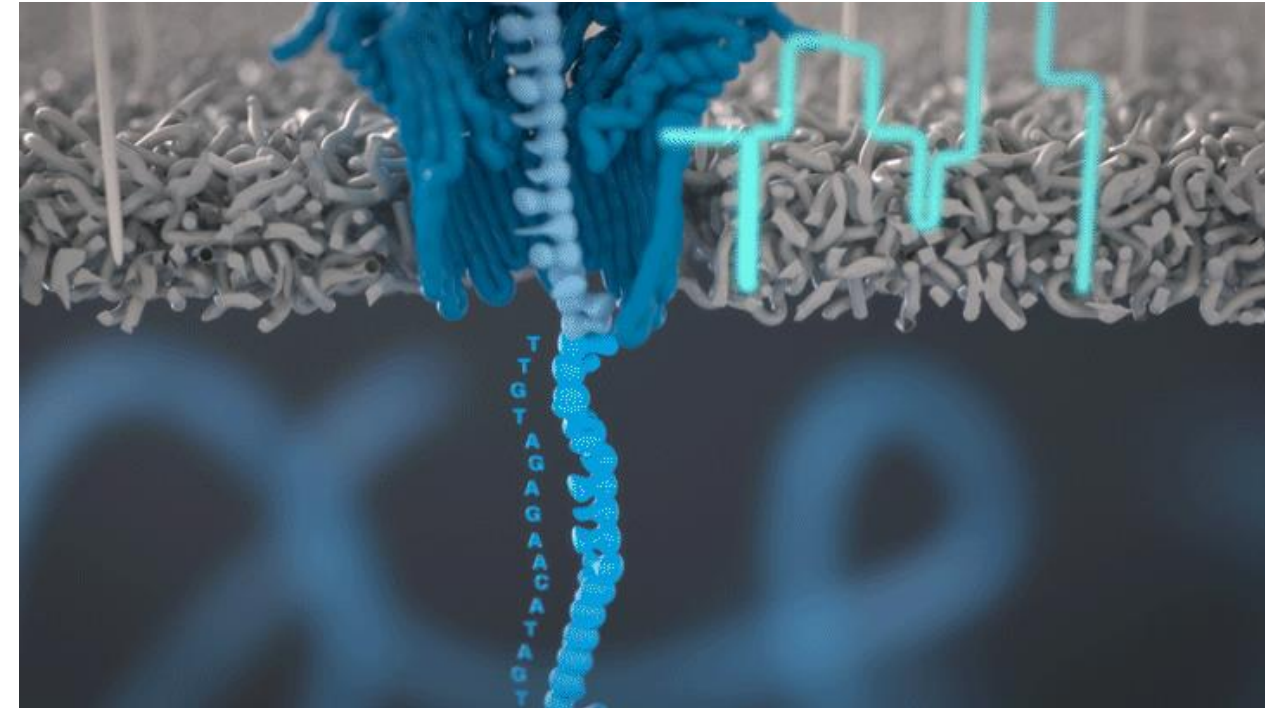
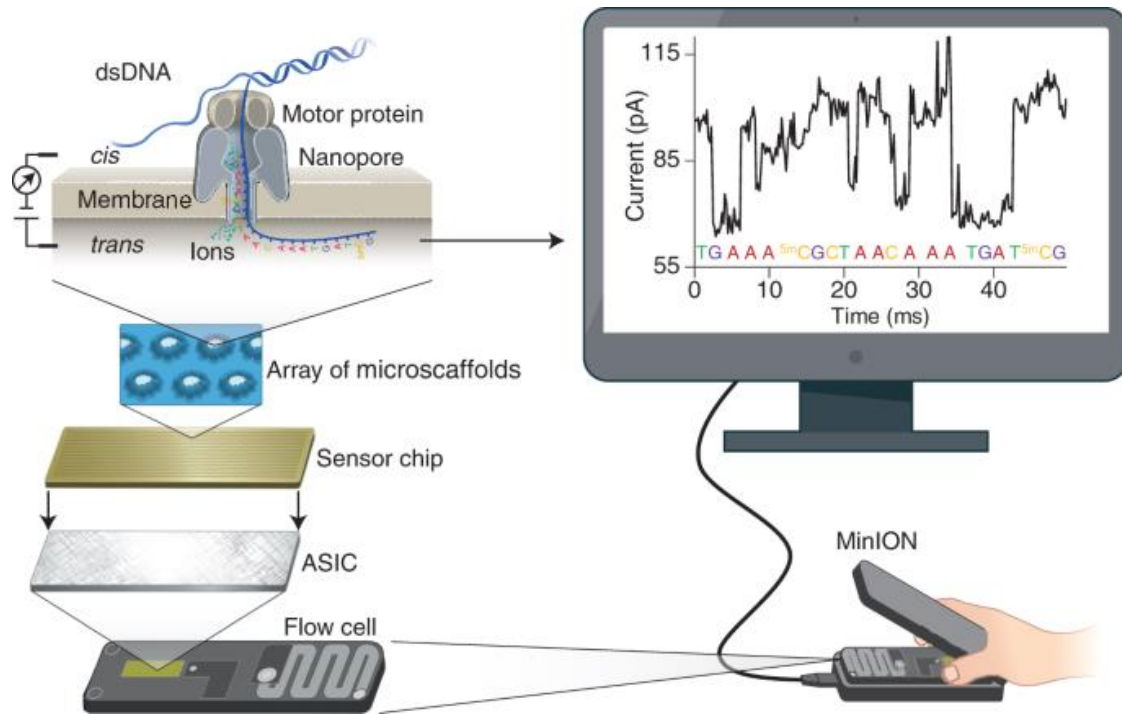


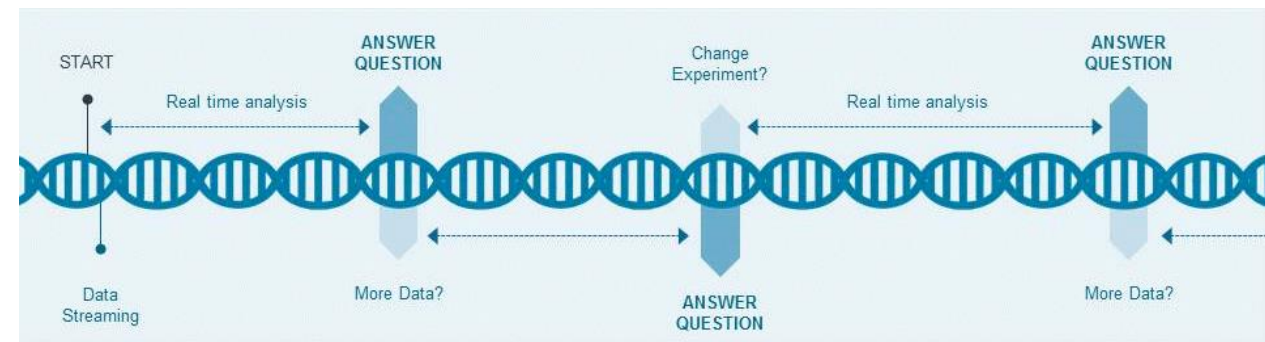
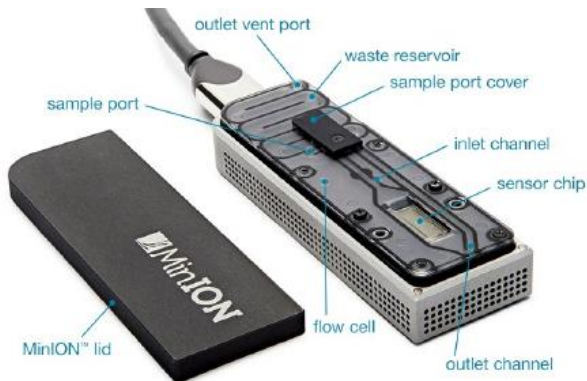
Figure 1-2-1 Pipeline of paired-end sequencing (www.illumina.com)



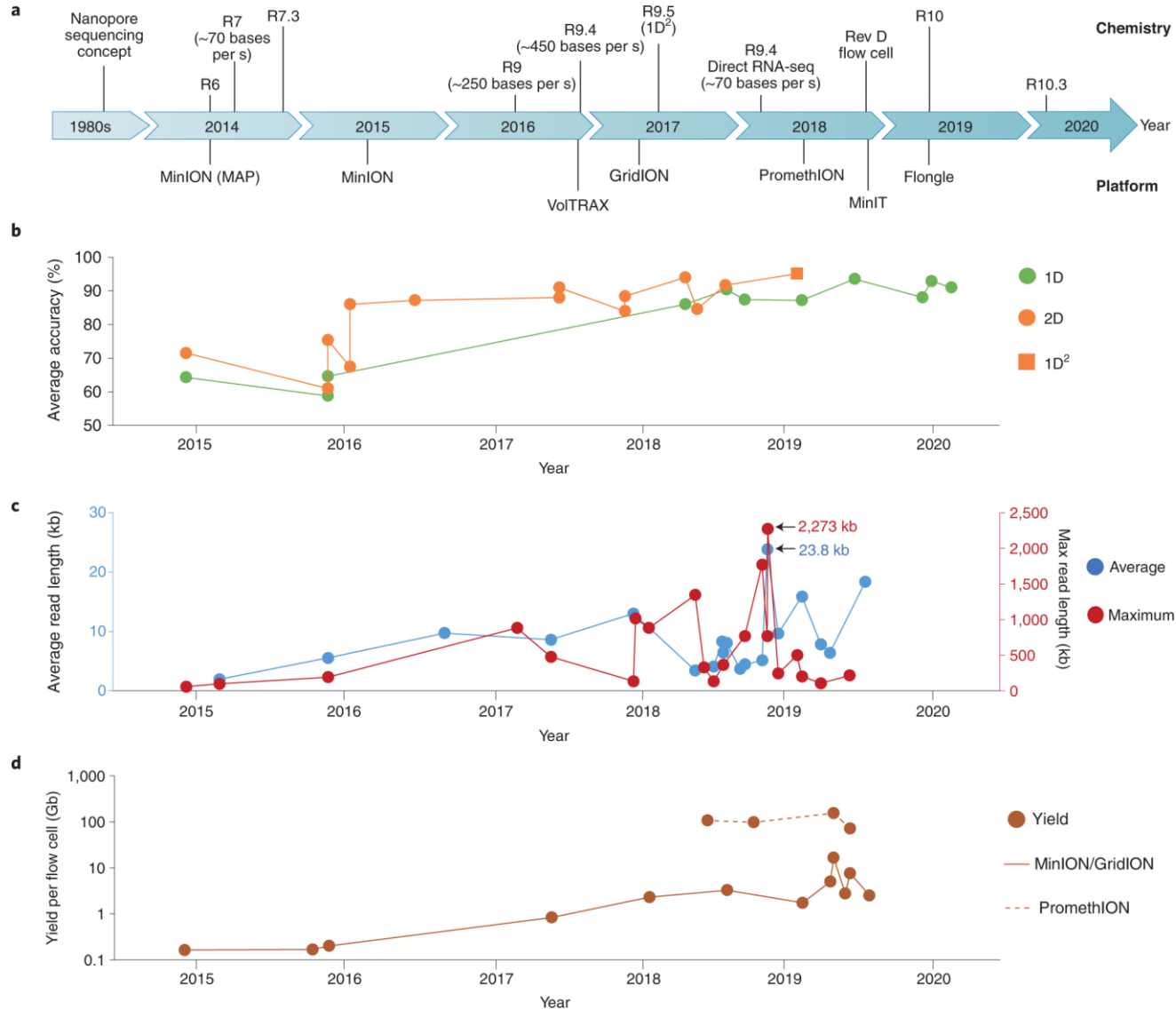
Oxford Nanopore: nanopore sequencing with real-time analysis



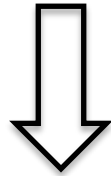
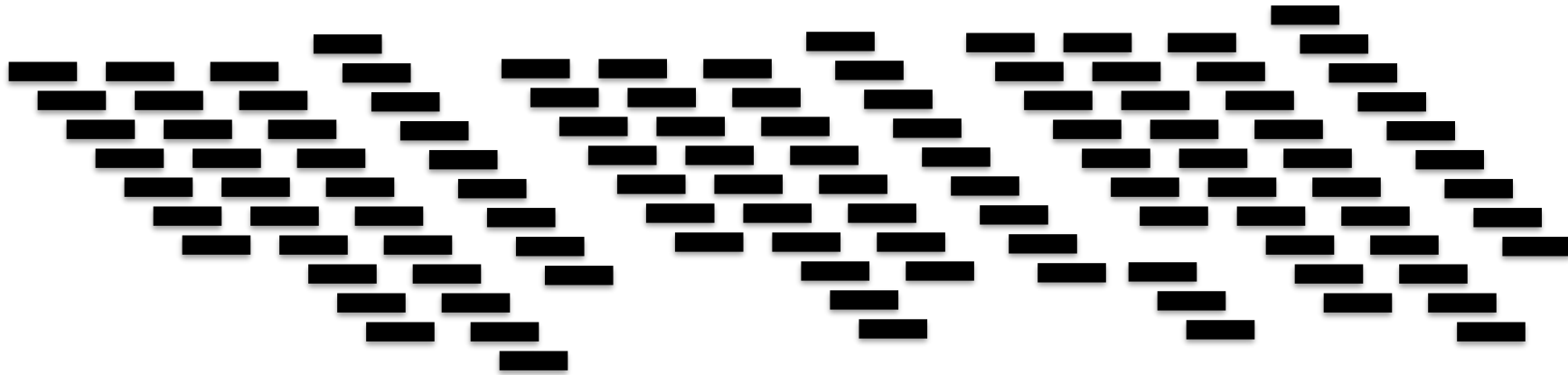
Wang et al 2021



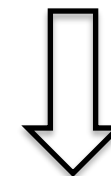
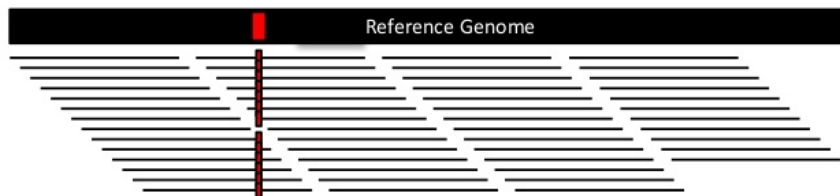
Oxford Nanopore: nanopore sequencing with real-time analysis



Two main approaches for handling reads...



Mapping or Reference Assembly

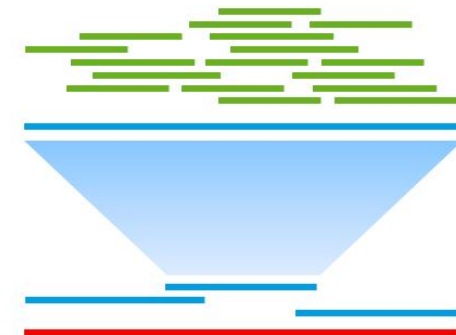


De novo Assembly

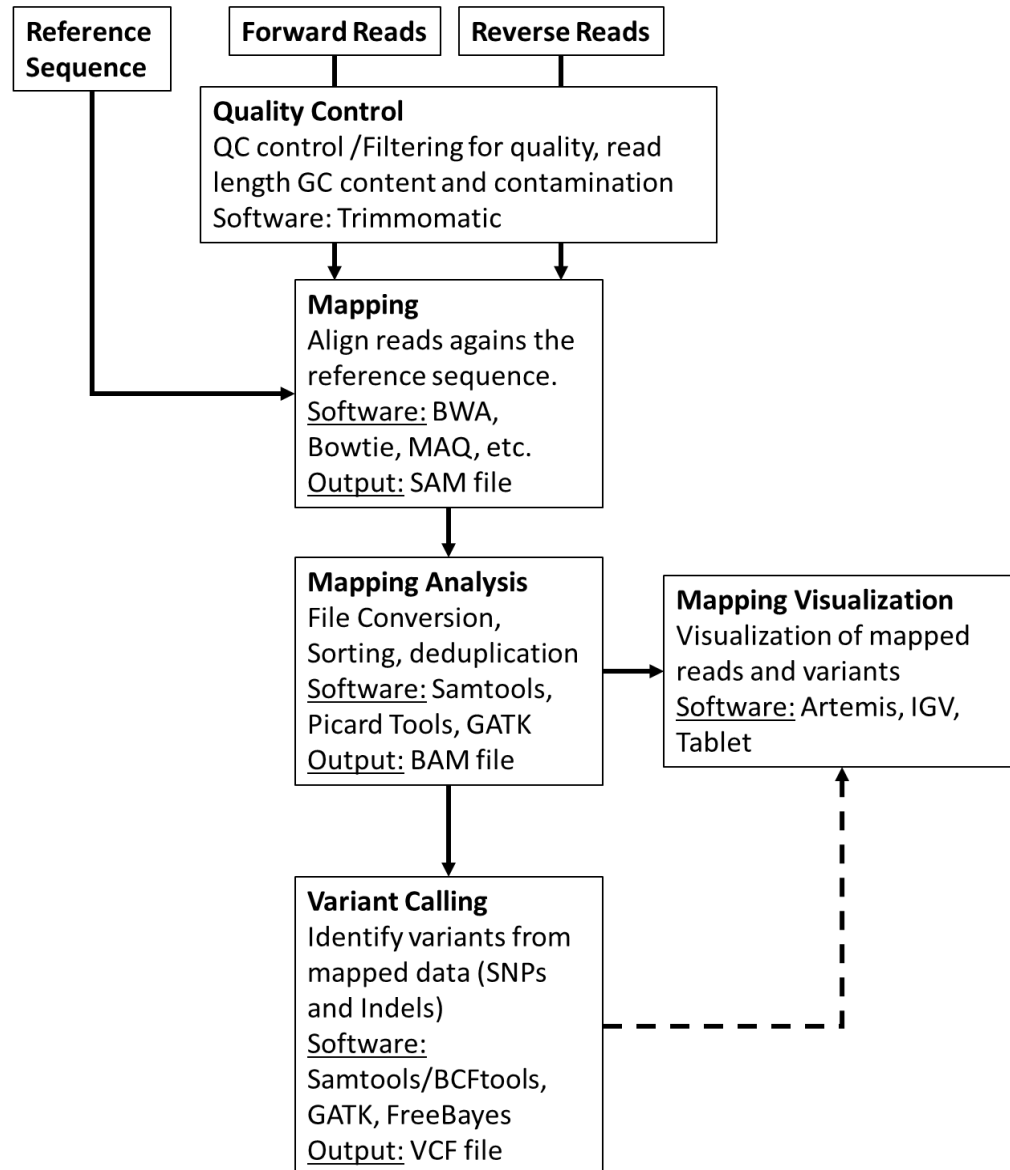
Short / Long reads

Contig assembly

Scaffold assembly
Finished genome



Workflow



Four main analytical stages:

- *Quality-control* – filter out reads/bases associated with poor basecall quality;
- *Mapping* – map reads to a reference genome, obtain sample coverage at each position and read coordinates;
- *Variant Calling* – identify variants existing between sequenced and reference genome, either SNPs or INDELs;
- *Functional Annotation* – determine the functional impact of each variant, e.g., which gene is affected? Is the mutation synonymous or non-synonymous? impact at the peptide primary structure?

What storage format for Sequencing Reads: FASTA vs FASTQ

FASTA

Label Title Line Comment

```
>fig|282458.1.peg.1 Chromosomal replication initiator protein dnaA
MSEKEIWEKVLEIAQEKL SAVSYSTFLKDTELYTIKDGEAIVLSSIPFNANWLNQOYAEI
IQAILFDVVGVEVKPHFITTEELANYSNNETATPKEATKPSTETTEDNHVLGREQFNAHN
TFDTFVIGPGRFPHAASLAVAEAPAKAYNPLFIYGGVGLGKTHLMAIGHHVLDNNPDA
KVIYTSSEKFTNEFIKSIRDNEGEAFRERYRNIDVLLIDDIQFIQNKVQTQEEFFYTFNE
LHQNNKQIVISSDRPPKEIAQLEDRLRSRFEWGLIVDITPPDYETRMALQKKIEEEKLD
IPPEALNYIANQIQSNIRELEGALTRLLAYSQLLGKPITTELTAELKDI IQAPKSKKIT
IQDIQKIVGQYINVRIEDFSAKKRTKS IAYPRQIAMYLSRELTD FSLPKIGE EFGGRDHT
TVIHAHEKISKDLKEDPIFKQEVENLEKEIRNV
```

Data Lines

FASTQ

Coordinates (x:y) and read

Lane

Tile

isfiltered

control number

index sequence

Instrument ID

Flow Cell ID

Run Number

```
@HWI-ST854:130:D17TLACXX:8:1101:7206:1827 1:N:0:TCGGCA
NAGCCTCCCACCCAGACCGCCCCGTAGCCAGCGCCTCGATTCTGTGGCCTGCTGGGGGGTGACGCCGCGTCCGAACGATCCGAATCGGCCGAGGTTAGG
+
#11ADDFHHBHHHIGGIIIIHHADHHIIIIIIIGAEGH: ?CEFD FCD@?@>ABCCBBB;;07<CBBCCB>>9@5<8<99<<BBBB7?(059599<98A89
@HWI-ST854:130:D17TLACXX:8:1101:7718:1835 1:N:0:TCGGCA
NGAGCGTGTATCCATGGCGGCGACACGCCGAACACCGTCGCCCTGAGCGCACGTTCCGGCGCCCAACGGCAGGGGCGAGCCGATATACGCCTCGCCGTCACCC
+
#1=DFFFFHHHHHJJIIJJIIJJIIJJJJHFFDCEDDDDDDDDDDDDDDBABDBDDDDDDDDDD?@BDBDB&0598?B7<<B@3<<<AD9<B#####
```

Sequence/quality line separator

sequence read

Sequence quality:

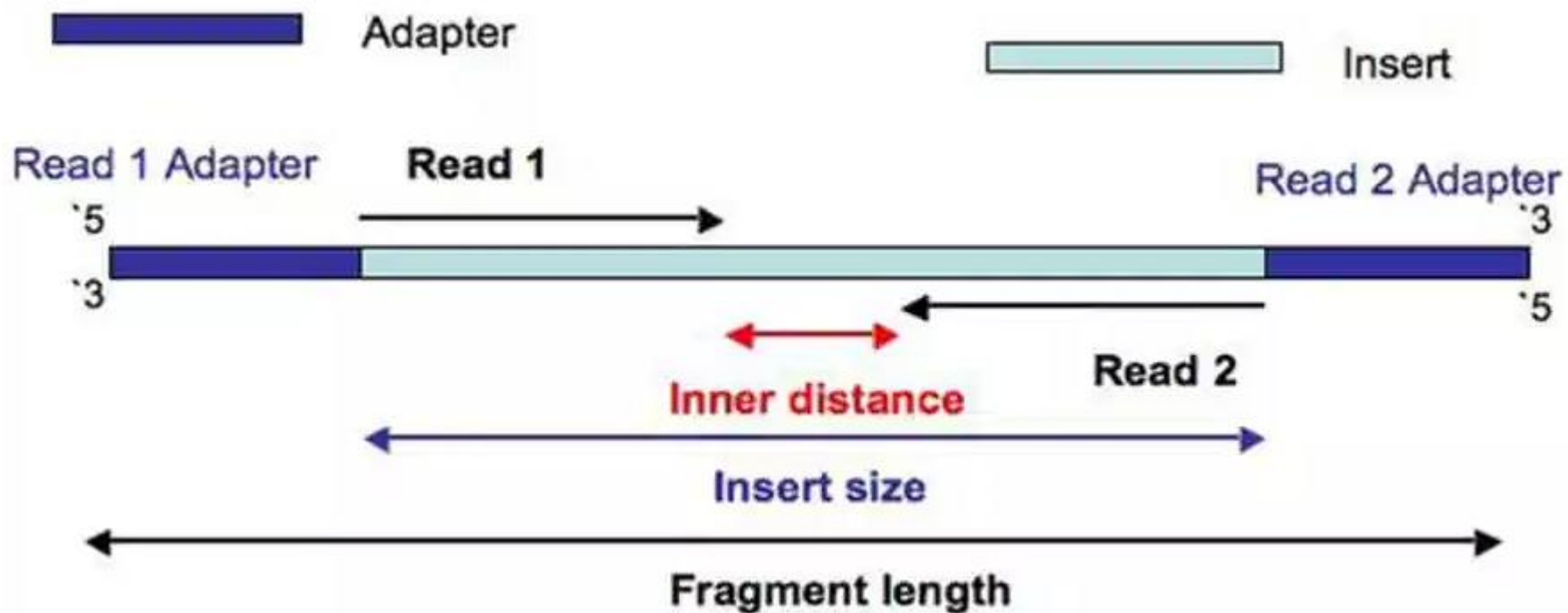
ASCII encoded Phred-33 quality score for each nucleotide of the sequence read above. Example: the 3rd nucleotide in the bottom read has a quality (Phred33 Q) of 28. Check the table below. What about the 5th nucleotide of the same read? _____

ASCII_BASE=33 Illumina, Ion Torrent, PacBio and Sanger

Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII
0	1.00000	33 !	11	0.07943	44 ,	22	0.00631	55 7	33	0.00050	66 B
1	0.79433	34 "	12	0.06310	45 -	23	0.00501	56 8	34	0.00040	67 C
2	0.63096	35 #	13	0.05012	46 .	24	0.00398	57 9	35	0.00032	68 D
3	0.50119	36 \$	14	0.03981	47 /	25	0.00316	58 :	36	0.00025	69 E
4	0.39811	37 %	15	0.03162	48 0	26	0.00251	59 ;	37	0.00020	70 F
5	0.31623	38 &	16	0.02512	49 1	27	0.00200	60 <	38	0.00016	71 G
6	0.25119	39 '	17	0.01995	50 2	28	0.00158	61 =	39	0.00013	72 H
7	0.19953	40 (18	0.01585	51 3	29	0.00126	62 >	40	0.00010	73 I
8	0.15849	41)	19	0.01259	52 4	30	0.00100	63 ?	41	0.00008	74 J
9	0.12589	42 *	20	0.01000	53 5	31	0.00079	64 @	42	0.00006	75 K
10	0.10000	43 +	21	0.00794	54 6	32	0.00063	65 A			

What storage format for Sequencing Reads: FASTA vs FASTQ

Why do I get two FastQ files?



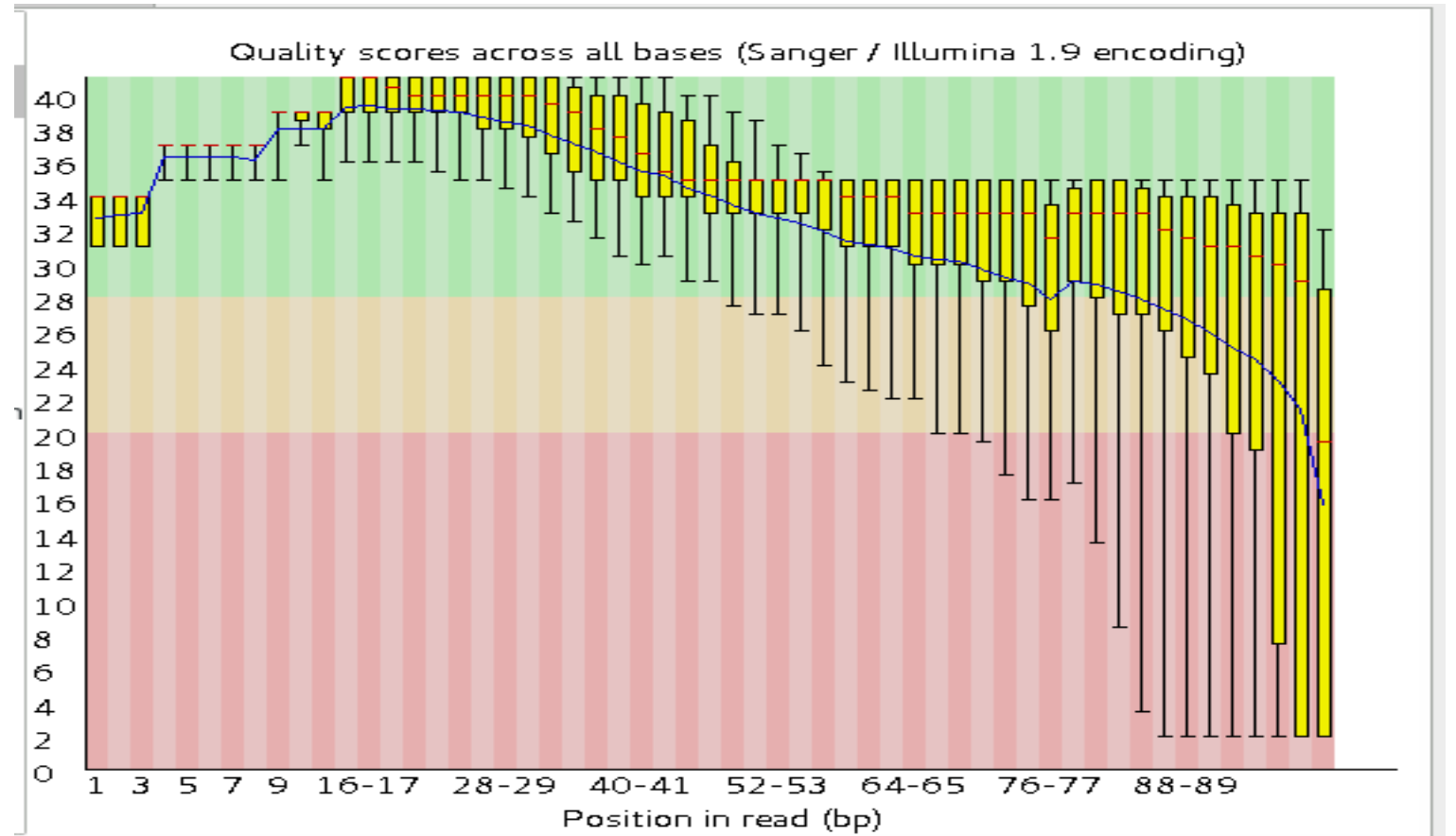
<https://thesequencingcenter.com/knowledge-base/what-are-paired-end-reads/>

Quality Control: Assessment

Objective:

- Assess overall sequencing quality and assess if sequencing metrics are within expected ranges;
- Remove base calls associated with low quality by removing or trimming sequencing reads;
- Taxonomical read QC – *did you sequence what you thought you did?*

Main/Most frequent problem: base quality deterioration along the read length



Quality Control: Assessment

How to assess sequencing metrics?

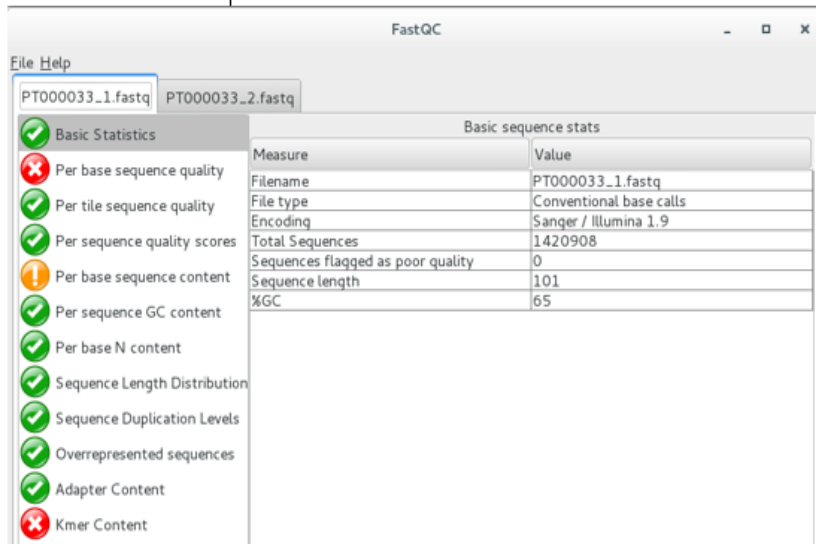
Software: FastQC, AfterQC, fastqp, HTSeq, etc.

FastQC – Java tool with both GUI and command-line as options.

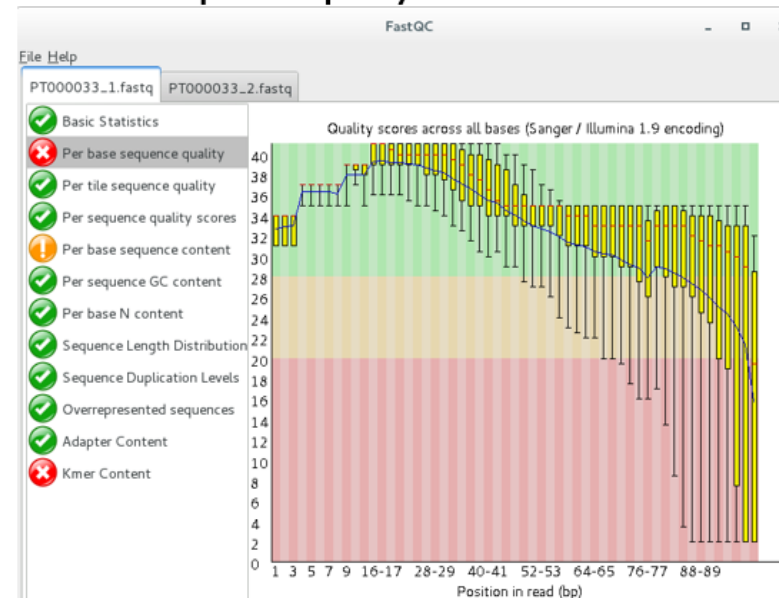
Input: FastQ files
(or SAM/BAM files)

Output: sequencing
metrics and plots

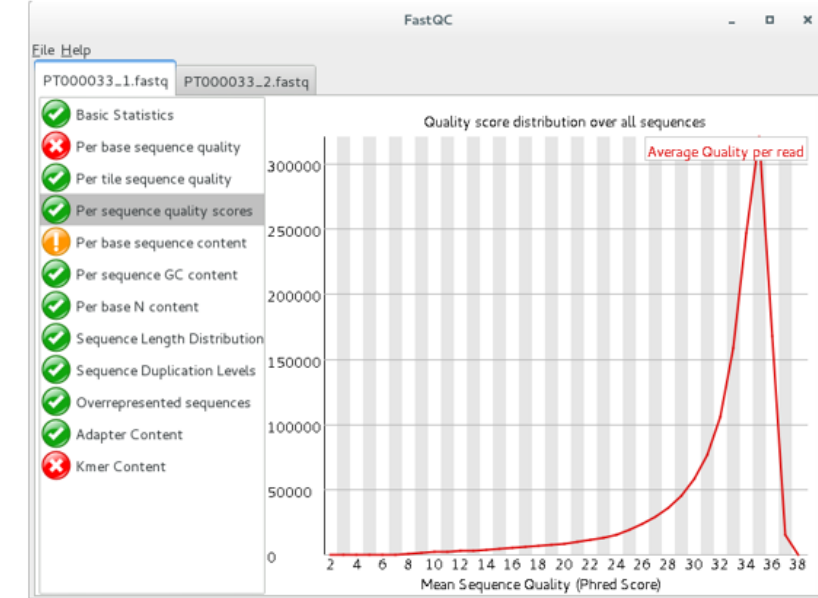
Basic Statistics



Per base sequence quality



Per sequence quality score



Quality Control: Assessment

How to assess sequencing metrics?

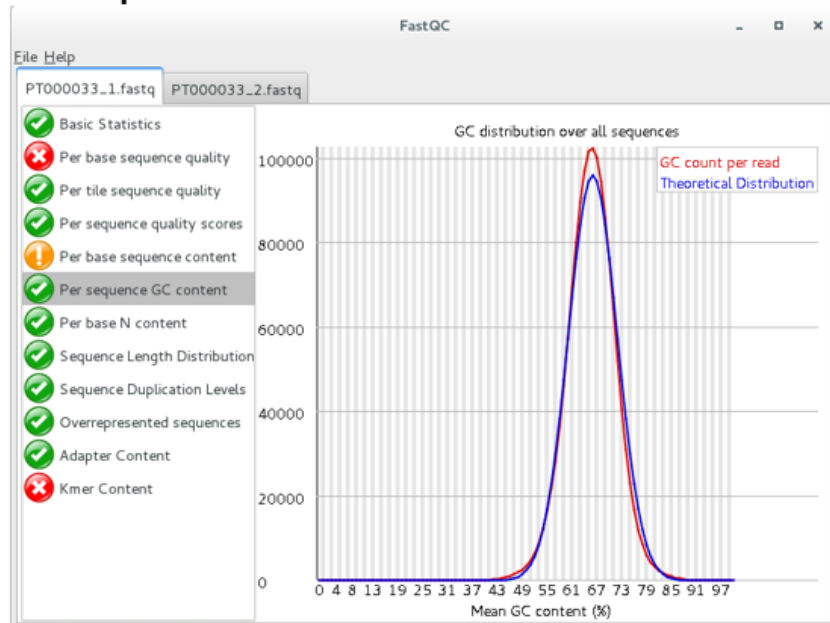
Software: FastQC, AfterQC, fastqp, HTSeq, etc.

FastQC – Java tool with both GUI and command-line as options.

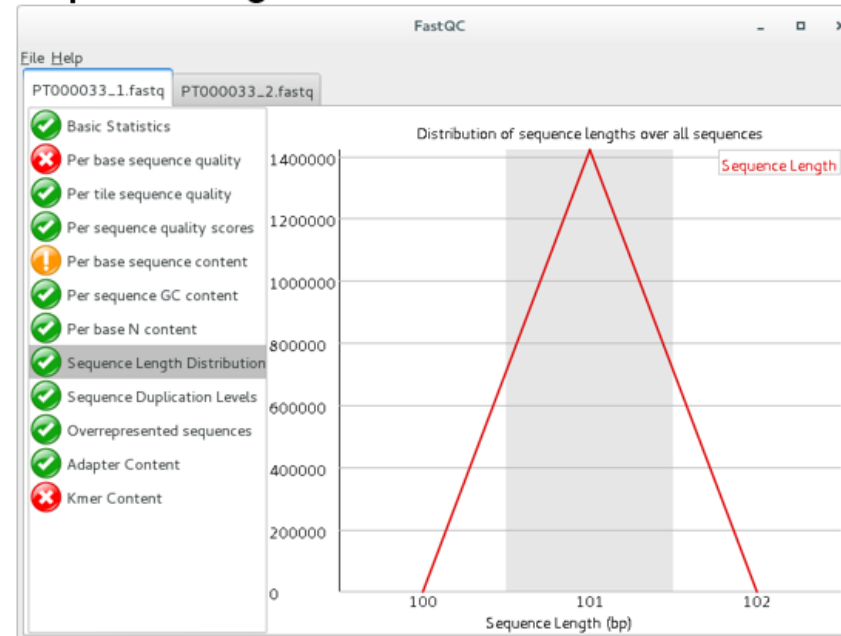
Input: FastQ files
(or SAM/BAM files)

Output: sequencing
metrics and plots

Per Sequence GC content



Sequence Length Distribution



Quality Control: Correction

How to correct, cut and filter out sequencing reads?

Software: Trimmomatic, FASTX, etc.

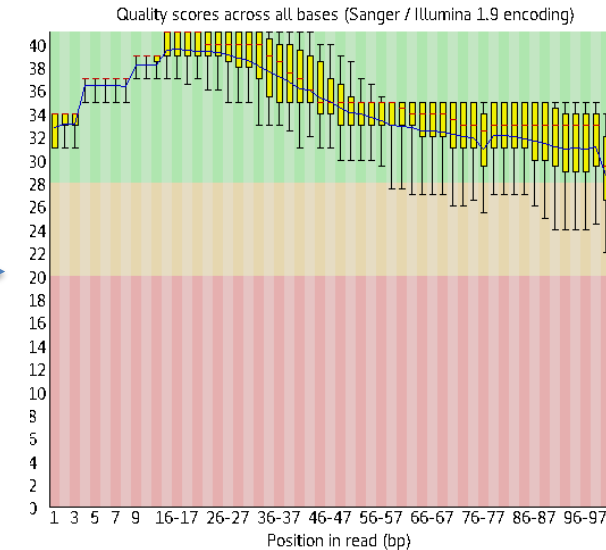
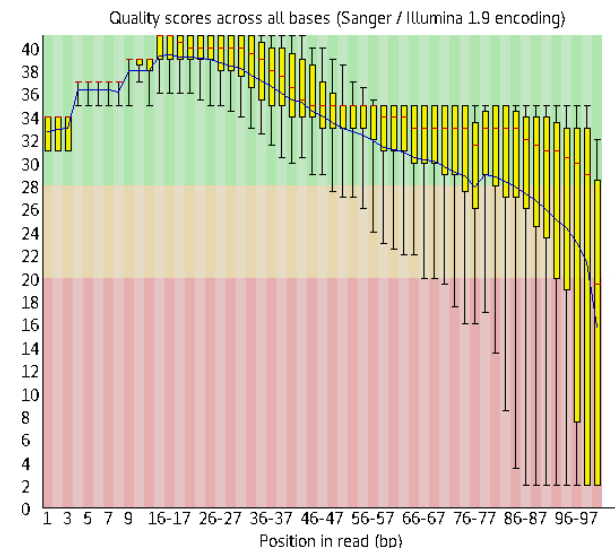
Trimmomatic – command-line Java tool capable of handling SE and PE reads.

Input: FastQ files.

Output: FastQ files with
trimmed/cut and surviving reads.

Trimmomatic can:

- Remove adapters;
- Remove leading and trailing low quality bases;
- Cut reads upon scanning at user defined sliding Windows when below a specified threshold;
- Remove reads when these don't meet a specified minimum length.



Quality Control: Taxonomical Read QC

Did I sequence what I thought I did? or Why doesn't it map? or Why does the assembly look strange?

Software: Kraken

Kraken – command-line tool that assigns reads to different taxonomical clades.

Input: FastQ files.

Output: Text Report.

0.89	28702	28702	U	0	unclassified	3.65	137472	137472	U	0	unclassified
99.11	3210397	42095	-	1	root	96.35	3631172	7288	-	1	root
97.81	3168297	1864	-	131567	cellular organisms	96.16	3623815	2151	-	131567	cellular organisms
97.76	3166433	16142	D	2	Bacteria	96.10	3621664	10917	D	2	Bacteria
97.25	3150142	4158	P	1224	Proteobacteria	95.81	3610594	13835	P	1224	Proteobacteria
97.12	3145930	19183	C	1236	Gammaproteobacteria	95.43	3596429	40211	C	1236	Gammaproteobacteria
94.74	3068819	83719	O	91347	Enterobacteriales	92.48	3485213	108814	O	91347	Enterobacteriales
92.16	2985015	1245831	F	543	Enterobacteriaceae	89.53	3374249	342184	F	543	Enterobacteriaceae
53.46	1731479	124411	G	561	Escherichia	79.20	2984764	1375209	G	570	Klebsiella
49.60	1606565	1603919	S	562	Escherichia coli	38.86	1464425	1415727	S	573	Klebsiella pneumoniae

Mapping or Reference Assembly

Objective: Find the origin of a sequencing read providing a reference genome is known

Reference genome: Should be a high quality genome, ideally finished, the close as possible to the sequenced genome.

Software: Burrows-Wheeler Aligner (BWA), Bowtie2, HISAT2

Input: FastQ files.

Output: Mapped/Alignment File
SAM/BAM file

Most mapping software implement the Burrows-Wheeler transformation algorithm which enables fast access to sequence data with an acceptable memory footprint.

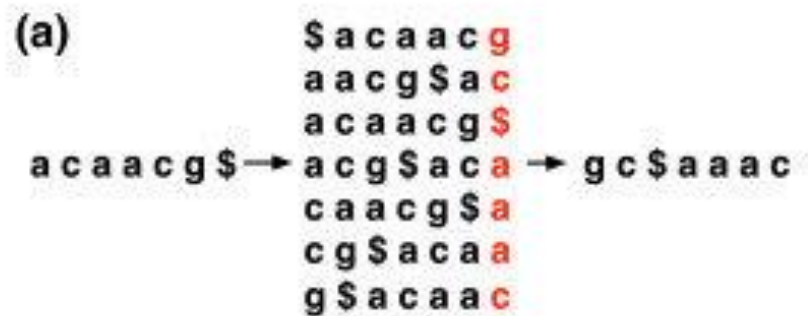
	Execution time				Memory usage				Accuracy				% Prop. paired reads			
	350		550		350		550		350		550		350		550	
Ins. (bp)	100	150	100	150	100	150	100	150	100	150	100	150	100	150	100	150
BWA	+	+	+	+	+	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++
Bowtie2	++	++	++	++	+++	+++	++	++	+++	+++	+++	+++	++	++	+++	+++
HISAT2	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	+	+

“We conclude that there is not a single mapper that is ideal in all scenarios but rather the choice of alignment tool should be driven by the application and sequencing technology.”

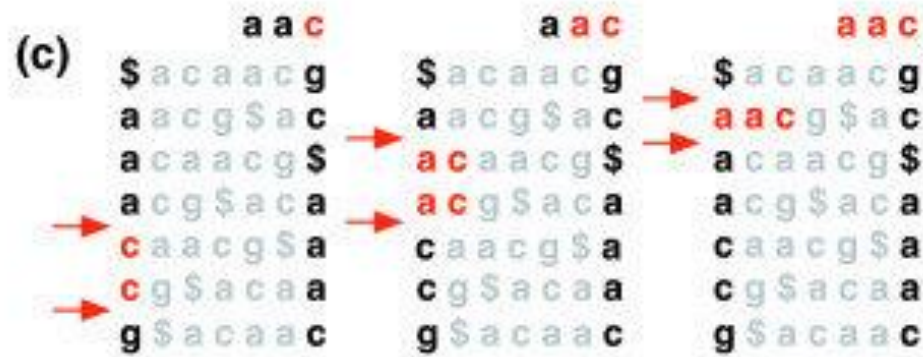
Keel et al 2018

The Burrows-Wheeler Transform

Reference compression:



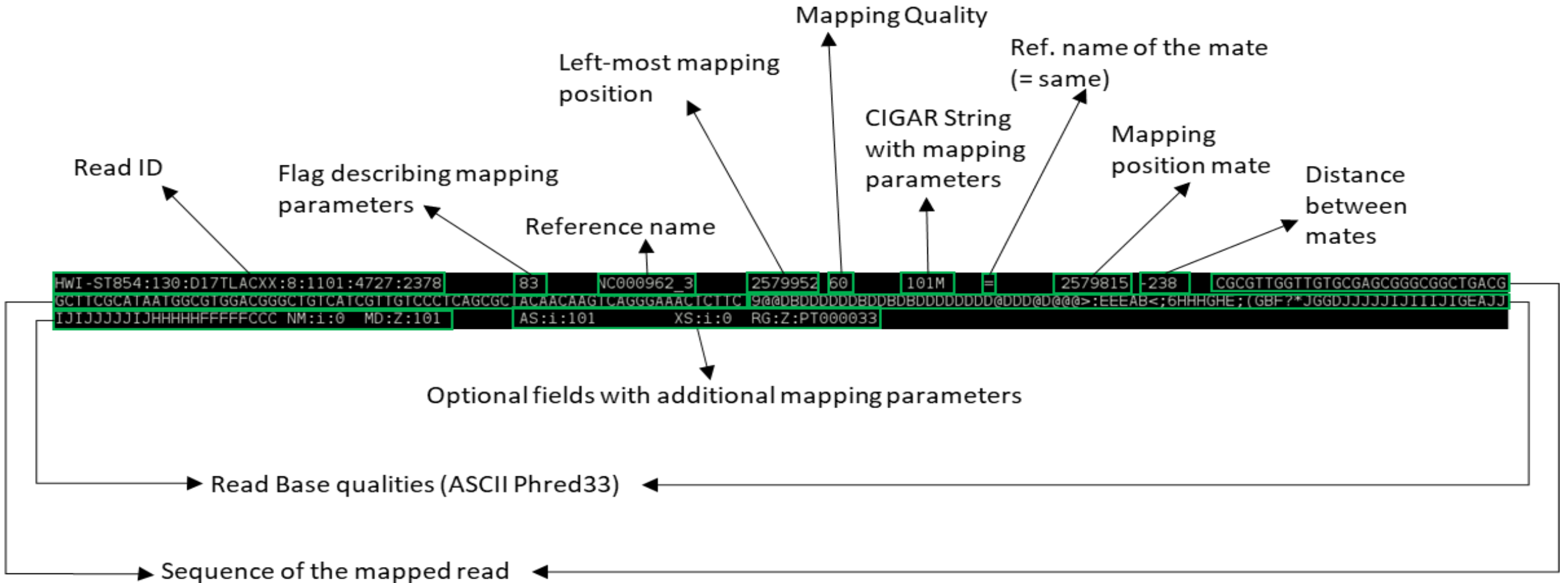
Searching for *aac* string:



Reconstructing original sequence:



The SAM Format...



The BAM files are a binary version of the SAM files (text format)

Both BAM and SAM files can be manipulated and viewed with SAMtools or Picard Tools

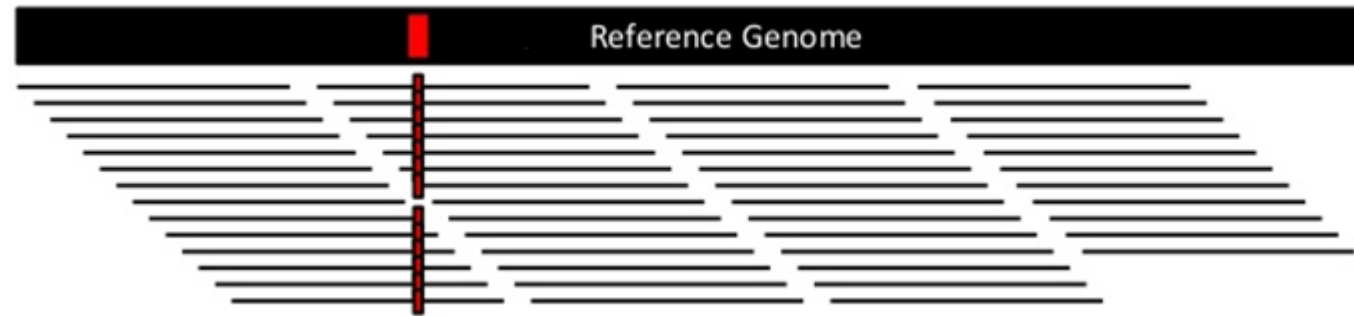
Variant Calling

Objective: Identify, list and store genomic variants, either SNPs or INDELs.

Software: SAMtools/BCFtools; Genome Analysis Toolkit (GATK), FreeBayes, LoFreq

Input: BAM/SAM files.

Output: VCF files



VCF Format

VCF header

```
##fileformat=VCFv4.0
##fileDate=20100707
##source=VCFtools
##reference=NCBI36
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality (phred score)">
##FORMAT=<ID=GL,Number=3,Type=Float,Description="Likelihoods for RR,RA,AA genotypes (R=ref,A=alt)">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##ALT=<ID=DEL,Description="Deletion">
##INFO=<ID=SVTYPE,Number=1,Type=String,Description="Type of structural variant">
##INFO=<ID=END,Number=1,Type=Integer,Description="End position of the variant">
```

Mandatory header lines

Optional header lines (meta-data about the annotations in the VCF body)

Body

CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	SAMPLE1	SAMPLE2
1	1	.	ACG	A,AT	.	PASS	.	GT:DP	1/2:13	0/0:29
1	2	rs1	C	T,CT	.	PASS	H2;AA=T	GT:GQ	0 1:100	2/2:70
1	5	.	A	G	.	PASS	.	GT:GQ	1 0:77	1/1:95
1	100	.	T		.	PASS	SVTYPE=DEL;END=300	GT:GQ:DP	1/1:12:3	0/0:20

Reference alleles (GT=0)

Alternate alleles (GT>0 is an index to the ALT column)

Deletion

SNP

Large SV

Insertion

Other event

Phased data (G and C above are on the same chromosome)

<http://vcftools.sourceforge.net/VCF-poster.pdf>

See full specs:

<https://samtools.github.io/hts-specs/VCFv4.2.pdf>

VCF Format

Format differences between variant callers - Examples

Finding allelic depth and filtering

SAMTools

NC000962_3 69871 . C T 225 . DP=23;VDB=0.641395;SGB=-0.69168;MQSB=0.537242;MQOF=0;AC=2;AN=2;DP4=0,0,11,8;MQ=43 GT:PL
1/1:255,57,0

```
bcftools view --include 'QUAL>=20 && INFO/DP>=10 && (INFO/DP4[2]+INFO/DP4[3])/(sum(INFO/DP4))>=0.9'
```

GATK

NC000962_3 69871 . C T 810 .
AC=1;AF=1.00;AN=1;BaseQRankSum=1.988;DP=24;Dels=0.00;FS=0.000;HaplotypeScore=0.9469;MLEAC=1;MLEAF=1.00;MQ=60.00;MQ0=0;MQRankSum=0.000
;QD=33.75;ReadPosRankSum=1.592;SOR=0.353 GT:AD:DP:GQ:PL 1:1,23:24:99:840,0

```
bcftools view --include 'QUAL>=20 && FORMAT/DP>=10 && (FORMAT/AD[*:1])/(FORMAT/DP)>=0.9'
```

Freebayes

NC000962_3 69871 . C T 656.091 .
AB=0;ABP=0;AC=1;AF=1;AN=1;AO=23;CIGAR=1X;DP=24;DPB=24;DPRA=0;EPP=3.10471;EPPR=5.18177;GTI=0;LEN=1;MEANALT=1;MQM=60;MQMR=60;NS=1;NU
MAL
T=1;ODDS=151.07;PAIRED=0.956522;PAIREDR=0;PAO=0;PQA=0;PQR=0;PRO=0;QA=760;QR=14;RO=1;RPL=14;RPP=5.3706;RPPR=5.18177;RPR=9;RUN=1;SAF=12;
SAP=3.10471;SAR=11;SRF=0;SRP=5.18177;SRR=1;TYPE=snp;technology.
illumina=1 GT:DP:AD:RO:QR:AO:QA:GL 1:24:1,23:1:14:23:760:-67.3027,0

```
bcftools view --include 'QUAL>=20 && FORMAT/DP>=10 && (FORMAT/AO)/(FORMAT/DP)>=0.9'
```

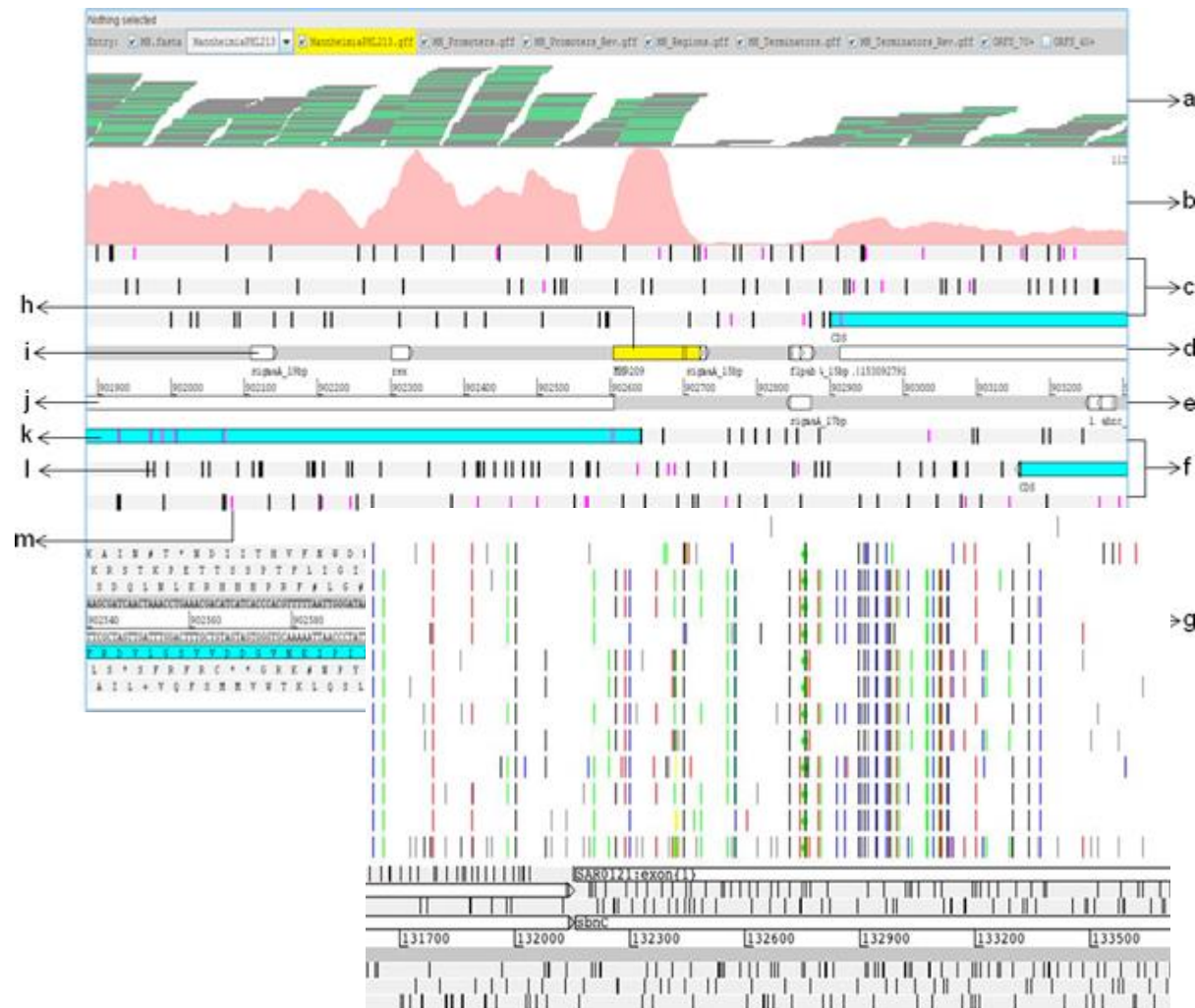
LoFreq

NC000962_3 69871 . C T 732 PASS DP=24;AF=0.958333;SB=0;DP4=0,1,12,11

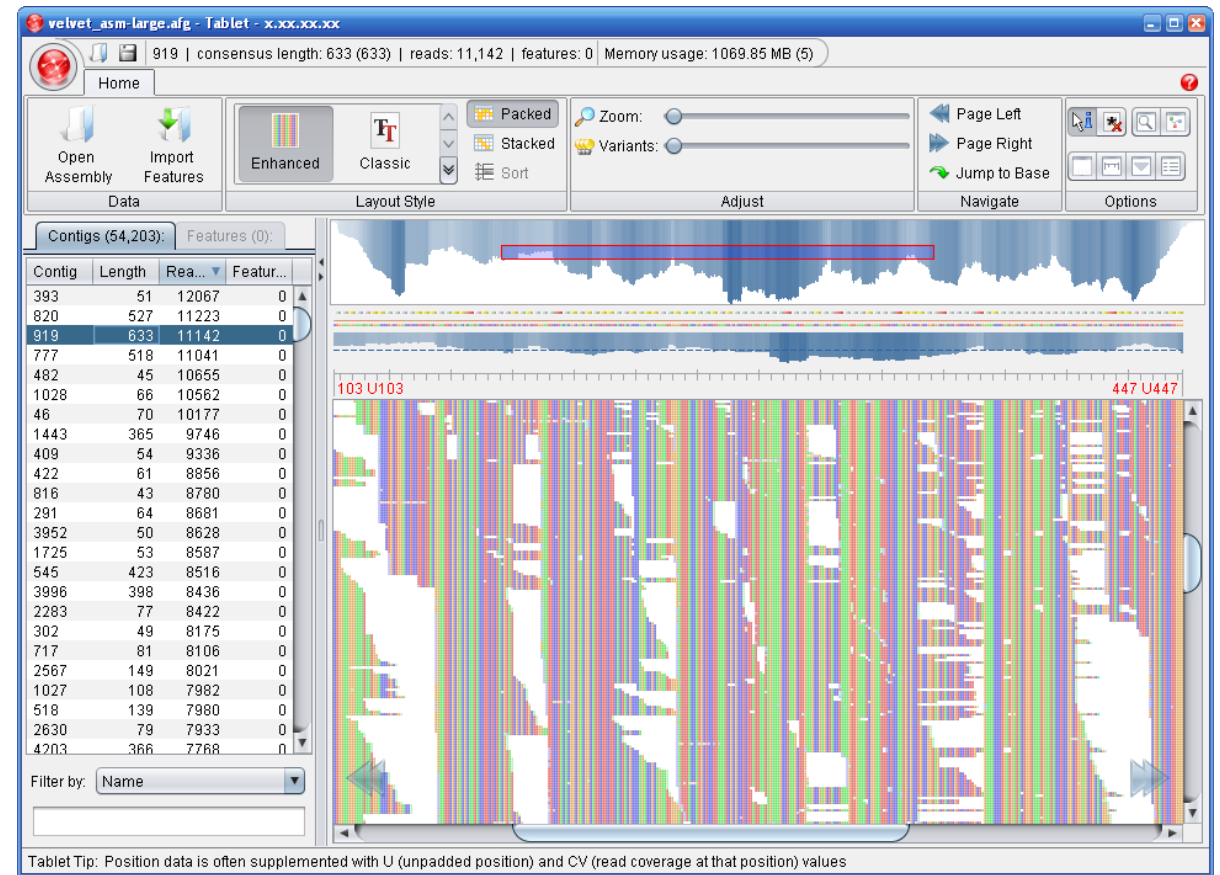
```
bcftools view --include 'QUAL>=20 && INFO/DP>=10 && (INFO/DP4[2]+INFO/DP4[3])/(sum(INFO/DP4))>=0.9'
```

BAM and VCF Visualization

Artemis



Tablet



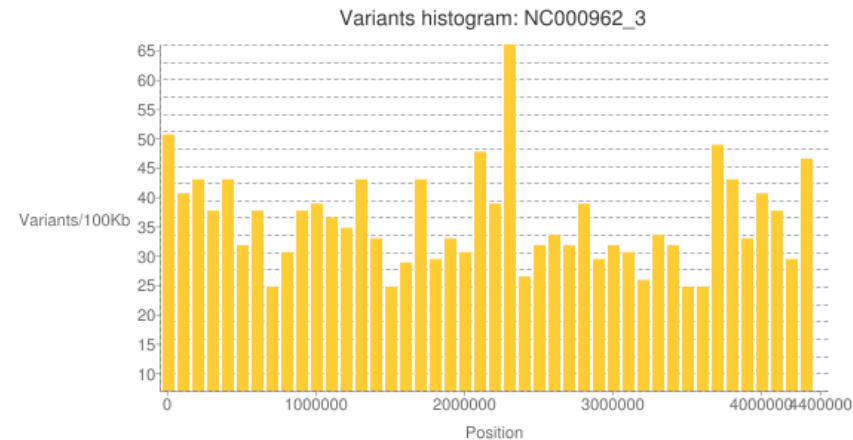
Functional Annotation

Objective: Annotate each variant with its functional impact/consequence.

Software: SnpEff, GATK etc

Input: VCF files.

Output: Annotated VCF files



Genome	NC000962_3
Date	2021-06-17 01:39
SnpEff version	SnpEff 5.0e (build 2021-03-09 06:01), by Pablo Cingolani
Command line arguments	SnpEff -no-downstream -no-upstream NC000962_3 PT000033.filt.vcf
Warnings	124
Errors	0
Number of lines (input file)	1,597
Number of variants (before filter)	1,598
Number of not variants (i.e. reference equals alternative)	0
Number of variants processed (i.e. after filter and non-variants)	1,598
Number of known variants (i.e. non-empty ID)	0 (0%)
Number of multi-allelic VCF entries (i.e. more than two alleles)	1
Number of effects	16,074
Genome total length	4,411,532
Genome effective length	4,411,532
Variant rate	1 variant every 2,760 bases

Number of effects by functional class

Type (alphabetical order)	Count	Percent
MISSENSE	786	61.025%
NONSENSE	13	1.009%
SILENT	489	37.966%

Missense / Silent ratio: 1.6074

'Allele | Annotation | Annotation_Impact | Gene_Name | Gene_ID | Feature_Type | Feature_ID | Transcript_BioType | Rank | HGVS.c | HGVS.p | cDNA.pos / cDNA.length | CDS.pos / CDS.length | AA.pos / AA.length | Distance | ERRORS / WARNINGS / INFO' ">

ANN=G|missense_variant|MODERATE|katG|Rv1908c|transcript|Rv1908c|protein_coding|1/1|c.944G>C|p.Ser315Thr|944/2223|944/2223|315/740||